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13. ABSTRACT (Maximum 200 Words) The purpose of this project is to identify and characterize examples of structural inheritance in yeast that are not prions. We described the first such phenomenon, [<i>Leu</i> ^P], and have made progress in this first year of funding in getting a nuclear genetic "handle" on its mechanistic basis by identifying strains that do not exhibit [<i>Leu</i> ^P]. Our most significant advance in this initial year has been the development of the peroxisome as a system to study structural inheritance. We have screened the yeast deletion collection to identify the entire set of genes required for maintenance of the peroxisome. These strains are then to be intercrossed to attempt to identify unlinked non-complementation of deletion alleles. If cytoduction reconstitutes a peroxisome in such doubly heterozygous deletion diploids, structural templating will have been demonstrated. If unlinked non-complementation is not observed for the peroxisome mutants, other structures of yeast will be examined for structural inheritance. The proposal presented initial experiments suggesting that the vacuole exhibits structural inheritance, but further work has not borne this out. In the course of that vacuole work, however, an interesting mating phenomenon was discovered which, although it doesn't bear on the issue of structural inheritance, will result in a publication.				
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INTRODUCTION:

The primary purpose of this proposal is to identify cases of structural inheritance using yeast as a model system. We believe that by developing a better understanding of the molecular events dictating "normal" cases of structural inheritance, we will ultimately generate solid underpinnings in which to think about aberrant cases leading to human disease such as those caused by prions. Two strains of genetically identical yeast differ phenotypically if a protein adopts the prion conformation in one and the non-prion conformation in the other. Thus, a structural difference, in this case due to the ability of certain proteins to adopt two folding states, one of which induces additional subunits of the protein to adopt that conformation, is manifest as a heritable difference. We have already identified the first case of structural inheritance in yeast not involving prions, termed the $[Leu^P]$ phenomenon (1). Our analysis of this phenomenon is described in detail below. Additionally, we have proposed an approach using unlinked non-complementation to identify novel cases of structural inheritance, specifically those in which cellular structures contain information required for formation of additional copies of this structure. We have made considerable progress on this Aim, examining two different yeast organelles for which there existed evidence that structural inheritance might be important. In one case, involving the yeast vacuole, we have not found evidence of structural inheritance and now believe that vacuoles are not likely to template themselves. Our studies of the vacuole did however prove the feasibility of our unlinked non-complementation approach and should lead to one or more manuscripts. We are now actively focusing on another non-essential yeast organelle, the peroxisome, and will focus on these preliminary findings in this report. Although, we have ruled out one potential case of structural inheritance in yeast during the first year of this proposal, we have succeeded in (1) demonstrating the efficacy of our screening approach, (2) generating novel findings concerning yeast vacuolar function and mating, and (3) defined the yeast peroxisome as a primary focus in our efforts to identify cases of structural inheritance in yeast.

BODY:

For purposes of clarity, we have divided the Body into three sections, one for each of the Aims presented in the proposal's "Statement of Work".

Aim 1. Characterize the molecular basis of $[Leu^P]$.

When a ρ^+ (respiratory-competent) strain completely loses its mitochondrial DNA, two types of ρ^0 strains can be readily obtained. One type of ρ^0 (DL156) grows as well as does its ρ^+ parent (strain 10507) while the other type of ρ^0 (DL035) grows two-fold more slowly on rich media and three-fold more slowly in the absence of leucine. Similarly, two types of ρ^- strains (which have lost large sections of their mitochondrial genome) can also be isolated from the same ρ^+ parent. What is the experimental basis for our conclusion that the heritable difference between DL156 and DL035, for example, is due to a change in mitochondrial structure? The simplest argument against nuclear specification of the slow growth, leucine-dependence phenotype is the high frequency with which this trait arises. A number of lines of evidence argue, instead, that the mitochondrion of the partially leucine-dependent strain suffered some as yet uncharacterized structural change. The most compelling evidence for an altered mitochondrial structure is the ability to convert, by cytoduction, a slow-growing, partially leucine-dependent strain to one that is normal growing, but only when mitochondria from a normal-growing strain are transferred. The heritable trait of the normal-growing ρ^0 and ρ^- derivatives is termed $[Leu^+]$ while that of the partially leucine-dependent strains is termed $[Leu^P]$. As stated, this represents the first case of structural inheritance in yeast not prion-related.

To date, we have not uncovered evidence that either a deficiency in Fe/S transport from the mitochondria or impaired pyruvate dehydrogenase is responsible for the $[Leu^P]$ phenotype. While we have not ruled out these possibilities, we have turned our focus to more unbiased approaches to define the molecular nature of $[Leu^P]$, as proposed in the event that neither of these possibilities proved to be correct. We describe these in detail below.

We have made a fundamental finding regarding the $[Leu^P]$ phenotypes that allows us to use standard yeast genetics to better define the phenomenon. Several yeast strain backgrounds were induced to become ρ^o by treatment with ethidium bromide. Unlike YDL121 or 16kar, in which ρ^o derivatives can be either $[Leu^+]$ or $[Leu^P]$, ρ^o derivatives from the WFalpha, W303 and FL200 backgrounds are always slow-growing and exhibit no dependence on leucine for growth. Thus, generation of $[Leu^+]$ ρ^o derivatives is strain-specific, implying that underlying polymorphisms exist in "wild-type" strain backgrounds that specify the ability of ρ^o derivatives either to exist in the $[Leu^+]/[Leu^P]$ states or to instead assume only a single ρ^o state. We intend to determine whether single gene differences dictate this difference and whether it is dominant or recessive. If the $[Leu^+]$ permissive phenotype is dominant, it will be possible to transform the WFalpha, W303 and FL200 strain backgrounds with an ARS-CEN library derived from YDL121 or 16kar, pool transformants, treat them with ethidium bromide to generate ρ^o derivatives and then select for $[Leu^+]$ isolates. This approach could lead to the identification of the genes that dictate $[Leu^+]$ permissiveness. Identifying the genes harboring relevant polymorphisms will likely provide meaningful clues to the mechanisms underlying the $[Leu^P]$ phenomenon.

Aim 2. Identification of new examples of structural templating using the yeast deletion strains.

We have decided that the peroxisome is an ideal organelle in which to examine structural templating. This organelle in *Saccharomyces* serves only a single function, the catabolism of fatty acids (β -oxidation) to yield acetyl-CoA. Its maintenance is therefore not required for viability. More than 25 so-called peroxins play roles in import of proteins into the organelle's matrix (Pex1, 2, 5, 6, 7, 8, 10, 12, 13, 14, 15, 17, 18, 20, and 21), regulation of organelle proliferation (Pex11, 25, 27, 28, 29, 30, 31, and 32), and, most relevant to this report, membrane protein insertion (Pex3, 19) (2).

There are several lines of evidence suggesting that the peroxisome may be incapable of self-assembly *i.e.*, that it exhibits structural inheritance. Most importantly, the alternative model, that the peroxisome is derived from the endoplasmic reticulum, is argued against by two lines of evidence: First, South et al. (3) have demonstrated that loss of Sec61p activity, which is well established to be a central component of the yeast ER translocon, has no effect on peroxisome biogenesis. Secondly, studies in animal cells (4,5,6) have shown that inhibition of COPI and COPII vesicular transport from the ER does not effect protein import into the peroxisome. Lastly, no pathway of membrane or phospholipid from the ER to the peroxisome in yeast or any other system has been identified, despite considerable effort. In other words, the peroxisome appears to lack interactions with any other organelle which would be providing the seed for the peroxisome's assembly.

The other line of evidence that a structural "seed" is responsible for creating the peroxisome comes from recent studies (7,8) on *pex3 Δ* and *pex19 Δ* strains. Prior to these recent studies it was established that deletion of either of these genes gave rise to yeast that were completely devoid of peroxisomes (9). However, the fact that re-supplying the wild type gene to the respective null strain allowed reformation of the peroxisome implied that the organelle can be formed *de novo*. The recent studies demonstrate that, on the contrary, *pex3 Δ* (8) and *pex19 Δ* (7) strains still contain peroxisomal remnants, as determined by biochemical and immunofluorescent

methods. These remnants, also termed “protoperoxisomes” (2), may in fact contain the seed which templates mature peroxisomes when *PEX3* or *PEX19* are re-introduced.

If a structure is indeed incapable of self-assembly and it contains a protein that is essential for the structure’s integrity, then removal of that protein should destroy the structure. Most importantly, re-introducing the protein should be incapable of reconstituting the structure because the information inherent in the structure would have been lost. In other words, the gene encoding such a protein should not be clonable by complementation.

The collection of yeast deletion strains (~4000 strains each deleted of a single non-essential gene) provides the ideal resource to identify such genes. Because the collection was constructed using knowledge of the sequence of the *Saccharomyces* genome rather than from the results of genetic complementation experiments, there is every reason for optimism that a subset of the deletions may not be clonable by complementation. The assumption that a self-templating structure instead has two such proteins allows us to employ the principle of unlinked non-complementation: haploid strains that are deleted in one or the other of such hypothetical proteins would each lack the structure. Mating them would create a doubly heterozygous deletion diploid which still lacked the structure. A phenotype that results from the lack of this structure would therefore be expected to be found, not just in the haploid mating partners, but in the diploid as well. Cytoduction to introduce the structure into the diploid would provide strong evidence that such a structure is responsible for templating itself.

Saccharomyces that lacks a functional peroxisome are unable to grow on plates containing oleic acid as the sole carbon source (10). We decided to screen the yeast deletion collection for strains that exhibit this phenotype. However, prior to collecting this data, a considerable amount of effort was directed at getting this plate assay to give reproducible data. This apparently has consistently been a problem in the field of *Saccharomyces* peroxisome biology (Paul Lazarow, personal communication) yet we seem to have finally arrived at a suitable formulation since we obtain consistent results. An additional obstacle is that yeast need to grow for a long period (2 weeks) on oleate plates.

Despite these obstacles, we have very recently been able to screen the yeast deletion strains for their inability to grow on oleic acid. Growth was scored relative to growth on identical media containing glycerol since the product of oleate metabolism, AcCoA, requires functional oxidative phosphorylation in order for it to be utilized as a energy source, as does glycerol. Table 1 summarizes these findings. The main reason we are confident that this screen was successful is that 14 of the 46 “large” effect genes that we identified have been previously established to cause an oleate requirement when deleted. Most of these 14 encode peroxins.

We have several strategies for culling this set of ~130 strains for those that, like *pex3Δ* and *pex19Δ* strains, contain no detectable peroxisomes. The most straightforward approach involves introducing into each of these strains a plasmid that encodes a Pex11-GFP fusion protein. A previous study (9) has established that the punctate fluorescent pattern exhibited by wild type cells, due to the localization of the GFP to peroxisomes, is abolished in *pex3Δ* and *pex19Δ* backgrounds. We are currently performing this analysis.

Once identification of the subset of the oleate growth-defective strains which lack peroxisomes is complete, we will test the MAT α version of each of these strains for these phenotypes (the screen was performed on the MAT α version of the deletion set). Finally we will intercross each of these strains to attempt to identify a doubly heterozygous deletion diploid that retains the phenotype of the haploid mating partners. If such a diploid is identified, cytoduction experiments as described above will be performed to attempt to reverse the phenotype. Such a reversal would be positive evidence for structural templating. Additional genetic and biochemical experiments would then be performed to attempt to determine the molecular details of this novel phenomenon.

The peroxisome is only one of potentially several structures in yeast that we can examine for structural templating. Because of our focus on other facets of this work in this first year of funding, we have not had the opportunity to identify other structures or organelles that could be examined using the scheme described above as applied to the peroxisome. We are currently in consultation with colleagues and brainstorming as to how to arrive at a list of candidate structures. The three criteria for selection of such structures are that they are non-essential, non-nuclear, and that they have a phenotype associated with their ablation.

Aim 3: The vacuole as a model for structural inheritance.

We had proposed in Aim 3 to use the yeast vacuole as a model for studying structural templating because it was reported to be a non-essential organelle (11), there are many easily assessed phenotypes arising from loss of a functional vacuole (12) and we had preliminary evidence for structural templating by crossing two vacuolar haploid mutants (*vps11Δ* and *vps16Δ*) which do not have a vacuole to obtain a doubly heterozygous diploid. This diploid was caffeine sensitive, consistent with no vacuolar function. Oddly, it also behaved as an α mater even though diploids are usually sterile. We performed follow-up analysis to determine whether this strain indeed contained a vacuole. However, to our surprise, using a commercial vacuolar stain, vacuoles were present in these cells and their morphology was not grossly altered. Our best guess is that this strain lost the copy of the chromosome that contains the wild type allele of *VPS11* or *VPS16* and also lost the copy of chromosome 3 that expressed MAT α . This strain would then express only MAT α and also be monozygous for either of the two *vps* deletions and thereby exhibit the observed phenotypes.

Although the particular vacuolar cross described above did not yield evidence of structural templating in the vacuole, it also did not rule out structural templating in this organelle. We reasoned the only way to get a reasonably accurate readout on whether structural templating is required for vacuolar formation would be to generate as complete a set as possible of the yeast genes required for vacuolar function. We examined several drug sensitivity phenotypes associated with vacuolar dysfunction, settling on sensitivity to high strontium levels in the medium as the most reproducible phenotype. Since known *vps* mutants are all strontium sensitive, we reasoned that this would be a suitable phenotype with which to screen the entire yeast gene deletion set. We have performed this analysis, leading to the identification of several known and unknown gene deletions that confer strontium sensitivity. We have intercrossed many of these sensitive strains, and to date, all doubly heterozygous diploids are strontium resistant, indicating that vacuoles appear to arise *de novo*, i.e., and that structural templating may not be occurring in this organelle. This conclusion is consistent with the myriad membrane trafficking in which the vacuole is involved.

We have also gone on to perform experiments on the strontium-sensitive mutants such as testing their sensitivity to other drugs. This should yield a manuscript of interest to the researchers studying vacuolar biogenesis. However, since structural templating does not seem to be involved in this aspect of yeast biology, we do not plan to continue our studies of the yeast vacuole, turning instead to the peroxisome and perhaps other structures as well (described above). Of course, structural templating may turn out to be rare, and our ability to find examples depends in part on the ability to screen through possible cases quickly.

Because of the odd mating behavior of the strain described above, we screened the non-essential yeast diploid gene deletion collection for diploids that retain the ability to mate. Several gene deletions have been discovered that allow mating in diploids, including a number involved in splicing mRNA derived from transcription of MAT α . While this study will also lead to a manuscript of interest, we are not pursuing it in detail because we again have no evidence of structural templating. One advantage of our methodology is that we are highly likely to generate

publishable information, no matter what phenotype we choose to study. However, we do not intend to focus large amount of our effort in these directions when structural templating is not apparent.

degree to which growth was impaired on oleate	cytoplasm	bud neck	cyto/nucleus	endosome	ER	Golgi	mitochondrion	nucleolus	nuclear periphery	nucleus	peroxisome	punctate composite	ambiguous	no assignment
LARGE	9	0	3	1	4	1	1	0	1	1	9	2	1	13
MEDIUM	12	1	5	0	1	0	5	1	0	8	1	0	3	5
SMALL	7	0	6	0	3	0	5	2	0	7	1	1	0	8

Table 1: Preliminary Results of the Oleate Screen of the Deletion Strain Array.

4668 MATa strains were pinned by robot onto two sets of plates, identical except for containing 0.1% oleate vs. 3% glycerol. 128 strains grew more poorly on oleate than on glycerol relative to most strains. Growth of each of these was retested by streaking onto the same oleate vs. glycerol media. The results after this retesting, presented above, are grouped according to the known cellular locations of the proteins encoded by the deleted genes as determined by the UCSF consortium (13). There are many unassigned localizations because the consortium did not establish the location of 51% of the non-essential proteins.

KEY RESEACH ACCOMPLISHMENTS:

- Identification of the [*Leu^P*] phenomenon as strain specific, leading to a genetic approach to identify the genes that underlie structural inheritance in the yeast mitochondrion.
- Proof of the efficacy and improvement of the use of unlinked non-complementation as a means of identifying cases of structural inheritance in yeast.
- Although we have largely ruled out the possibility that the yeast vacuole contains structural information, we have made a number of discoveries regarding yeast vacuolar function that will lead to a manuscript.
- Discoveries relating to yeast mating that will lead to a manuscript.
- Identification of new genes important for peroxisome biosynthesis that may form protein complexes containing structural information.

REPORTABLE OUTCOMES:

Databases that will be made public in upcoming manuscripts

- Database of yeast gene deletions sensitive to strontium.
- Database of yeast gene deletions required for growth in the presence of oleic acid.
- Database of yeast gene deletions that permit mating of diploids.

Manuscripts

- Schmidlin, T., Kaeberlein, M., Lockshon, D. and B.K. Kennedy. Genome-wide screen identifies genes in yeast that are required to maintain stability in diploids. Manuscript in preparation.

Presentations

- XXI International Conference on Yeast Genetics and Molecular Biology 2003, Gothenburg, Sweden, Dan Lockshon, Poster presentation.
- 2004 Yeast Genetics and Molecular Biology Meeting (July 27-31), Seattle, Dan Lockshon, Oral presentation and Poster presentation

CONCLUSIONS:

Although we have been unable thus far to identify new cases of structural inheritances in yeast, we have optimized the unlinked non-complementation approach to identify such cases in our first year of funding. We have also made discoveries regarding (1) yeast vacuolar biology, (2) yeast peroxisome biology (3) yeast mating and (4) better characterized the yeast [*Leu^P*] phenomenon. As stated in our proposal, the totality of biological information is larger than the information contained in the genome. We believe that, while our undertaking to identify new cases of structural inheritance may be difficult, it will ultimately lead to a better understanding of how cells are organized and provide a backdrop through which to better understand the derivation and mechanism of action of prions.

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